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Presence of small GTP-binding proteins in the peroxisomal membrane

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Highly purified peroxisomal membranes stripped from their peripheral membrane proteins and only minimally contaminated with other membranes, contained three GTP-binding proteins of 29, 27 and 25 kDa, respectively. Bound radioactive GTP was displaced by unlabelled GTP, GTP analogs and GDP but not by GMP or other nucleotides. GTP binding was markedly decreased by trypsin treatment of intact purified peroxisomes; it increased 2–3-fold after pretreatment of the animals with a peroxisome proliferator. We conclude that the peroxisomal membrane contains small GTP-binding proteins that are exposed to the cytosol and that are firmly anchored in the membrane. We speculate that these proteins are involved in peroxisome multiplication by fission or budding during peroxisome biogenesis and proliferation.

Introduction

Over the last few years a large number of GTP/GDP-binding proteins has been discovered in eukaryotic cells. At least 30 of them are low molecular mass (20–30 kDa) monomeric proteins that are structurally related to the ras oncoprotein (for reviews, see Refs. 1–5). Although no definitive function has been assigned to these proteins, they are believed to be involved in controlling cell growth, differentiation and transformation, cytoskeletal organization and vesicular transport via the exocytotic and endocytotic pathways. In the latter process they seem to play a role in vesicle budding, targeting and fusion. Accordingly, small GTP-binding proteins have been identified in the endoplasmic reticulum [6], the Golgi compartments [7,8], secretion granules [9–13], plasma membrane [7], endosomes [7] and clathrin-coated vesicles [14]. Besides being present in the membranes of compartments involved in exocytosis/endocytosis, they also have been found in the nuclear envelope [15] and in the cytosol [12,16].

Peroxisomal matrix and membrane proteins are synthesized on free polyribosomes in the cytosol. They are thought to be imported in pre-existing peroxisomes or perhaps properoxisomes which multiply by fission or budding [17]. A unique feature of peroxisomes is their capacity to proliferate (reviewed by Hawkins et al., [18]). For instance, the number of hepatic peroxisomes can increase up to 10-fold after treatment of rodents with hypolipidemic fibrates or other peroxisome proliferators. Recently, Ohno and Fuji [19] described the presence of peroxisome-forming sheets in cultured mouse hepatocytes treated with the peroxisome proliferator clofibrate. The peroxisome-forming sheets occurred as smooth membranous structures from which multiple peroxisomes were budding. Since small GTP-binding proteins have been implicated in vesicle budding and fusion in the exocytotic/endocytotic pathways, we investigated the presence of such proteins in the peroxisomal membrane. Our results indicate that three such proteins are present. We speculate that they play a role in peroxisome biogenesis and proliferation.

Materials and Methods

Animals. Male Wistar rats weighing 200–250 g were used. They were maintained on a standard laboratory chow diet. Clofibrate-treated rats were kept on a standard diet containing 0.3% (v/w) clofibrate for 2 weeks.

Cell fractionation and purification of peroxisomes. Liver homogenates (25%, w/v) prepared in 0.25 M sucrose, 0.1% (v/v) ethanol and 1 mM dithiothreitol (homogenization solution) were fractionated by differential centrifugation as described by Mannaerts et al.

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Abbreviations: GTPγS, guanosine 5'-[γ-thio]triphosphate; p[NH]ppG, guanosine 5'-[β,γ-imido]triphosphate; Mops, 3-(*N*-morpholino)propanesulfonic acid.

[20]. The λ-fractions, enriched in lysosomes and peroxisomes, were subfractionated by isopycnic centrifugation in iso-osmotic self-generating Percoll gradients [21]. The gradient solution contained 40% (w/v) Percoll (Pharmacia, Sweden), 0.22 M sucrose, 1 mM dithiothreitol, 1 mM Mops, 1 mM EDTA and 0.1% (v/v) ethanol. Approx. 3 ml of a λ-fraction, resuspended in homogenization solution and derived from 10 g of liver, was layered on top of 36 ml of the Percoll solution and centrifuged at 4°C for 1 h at 34000 × *g* in a MSE 8 × 50 ml fixed-angle rotor. The gradient was collected in 15 fractions of equal volume, starting from the bottom. Fractions 8–12, containing approx. 50% of the catalase of the gradient, were pooled, diluted 4.5-fold with homogenization solution containing 1 mM EDTA (pH 7.2) to lower the Percoll concentration and centrifuged for 24 min at 12300 × *g*. The loosely packed pellet was 3.5-fold diluted with the homogenization medium containing EDTA and centrifuged for 12 min at 1700 × *g*. The supernatant was centrifuged for 24 min at 12300 × *g*, resulting in a pellet which contained 60% of the peroxisomes present in the pooled fractions, but only 20% of the contaminating microsomes. The peroxisomes obtained by this method are referred to as Percoll-purified peroxisomes. In some experiments, the Percoll-purified peroxisomes were resuspended in the homogenization solution containing EDTA (peroxisomes derived from 8 g of liver in 1 ml) and further purified by centrifugation in a Nycodenz step gradient. Approx. 2 ml of the peroxisome suspension was layered on top of 16 ml of 30% (w/v) Nycodenz solution ($d = 1.15$), which rested on 4 ml of 45% (w/v) Nycodenz ($d = 1.24$) and a cushion of 2 ml of 56% (w/v) Nycodenz ($d = 1.30$). All Nycodenz solutions contained 5 mM Mops (pH 7.2), 0.1% (v/v) ethanol and 1 mM EDTA. Gradients were prepared in 25-ml thick-walled polycarbonate tubes and centrifuged in a Beckman 55.2 Ti rotor at 130000 × *g* for 1 h with slow acceleration and deceleration rates.

Peroxisomal membranes were prepared by sonication of the purified organelles in 10 mM pyrophosphate buffer (pH 9) or by suspension in 0.1 M Na_2CO_3 buffer (pH 11), as described by Van Veldhoven et al. [21].

In a number of experiments, the whole cell fractionation procedure was carried out in the presence of the proteinase inhibitors benzamidine (5 mM), phenylmethylsulfonylfluoride (200 μM) and *N*-tosyl-L-phenylalaninechloromethane (50 μM). The presence of proteinase inhibitors did not affect the apparent molecular mass of the three peroxisomal GTP-binding proteins.

Determination of marker enzymes and protein. Marker enzymes (glucose-6-phosphatase and esterase, endoplasmic reticulum; catalase, peroxisomal matrix; glutamate dehydrogenase, mitochondrial matrix; acid phosphatase, lysosomes) were determined as described previously [22]. 5'-Nucleotidase (plasma membrane) and galactosyltransferase (Golgi apparatus) were measured as described by Van Veldhoven et al. [23] and Van Veldhoven and Mannaerts [24], respectively.

Protein was determined according to the method of Peterson [25] with bovine serum albumin as standard.

SDS-PAGE. SDS-PAGE was performed in 10–20% (w/v) acrylamide gradient slab gels with a 4% (w/v) acrylamide stacking layer, essentially as described by Laemmli [26], in a vertical electrophoresis system. The proteins were then transferred to nitrocellulose membranes by the method of Kyshe-Andersen [27]. After blotting, the gels were stained with Coomassie blue [28]. The proteins on the nitrocellulose membranes were briefly visualized by Ponceau S staining [29] before incubation with [α-^{32}P]GTP and stained with amido-black [30] after incubation with [α-^{32}P]GTP.

GTP-binding assay. After blotting, the nitrocellulose sheets were incubated with [α-^{32}P]GTP, essentially as described by Lanoix et al. [6], washed and autoradiographed at −80°C with the aid of Kyokko Speed Super High 500 intensifying screens (Kyokko, Japan). Bound radioactive GTP was quantified by means of an LKB Ultrascan XL enhanced laser densitometer (LKB, Sweden).

Materials. [α-^{32}P]GTP and [α-^{32}P]ATP (spec. act. 3000 Ci/mmol) were from Dupont NEN Research Products, Boston, MA, USA; unlabelled nucleotides and analogs were from Boehringer-Mannheim, Germany. Nitrocellulose membranes with a pore size of 0.2 μm were obtained from Schleicher and Schuell, Dassel, Germany.

Results

In order to investigate the presence of GTP-binding proteins in the peroxisomal membrane, a λ-fraction prepared by differential centrifugation and enriched in peroxisomes was further purified by isopycnic centrifugation in an iso-osmotic self-generating Percoll gradient. Fractions 8–12 of the Percoll gradient (Fig. 1), which contained most of the catalase (peroxisomal marker) and only a small fraction of glucose-6-phosphatase and esterase (endoplasmic reticulum), 5'-nucleotidase (plasma membrane) and galactosyltransferase (Golgi apparatus), were pooled and centrifuged. The sedimented peroxisomes were washed by centrifugation as described in Materials and Methods, which resulted in a further loss of contaminating organelles. Table I gives the composition of the λ-fraction and of the final peroxisomal preparation (Percoll-purified peroxisomes). Calculated on a protein basis, the Percoll-purified fraction of Table I contained 6–10% microsomes (endoplasmic reticulum). This calculation is based on: (1), the relative specific activities of the endoplasmic reticulum markers glucose-6-phosphatase

and esterase and (2), the assumption that 20% of the total liver protein belongs to the endoplasmic reticulum [31]. Thus, a relative specific activity of 1 for an endoplasmic reticulum marker corresponds to a contamination by endoplasmic reticulum of 20% on a protein basis; relative specific activities of 0.3 (glucose-6-phosphatase) and 0.5 (esterase) correspond to a contamination of 6 and 10%, respectively. The Percoll-purified peroxisomes were then sonicated in hypotonic pyrophosphate buffer, in order to release the matrix proteins. The peroxisomal membranes were sedimented by centrifugation, solubilized with sodium dodecyl sulfate and the membrane proteins were separated by SDS-PAGE. The polypeptides were then blotted onto nitrocellulose sheets and incubated with [α-^{32}P]GTP. The autoradiograph in Fig. 2 shows GTP-binding to a group of polypeptides in the 25–30 kDa range. Bound radioactive GTP was displaced by unlabelled GDP but not by GMP, as is to be expected for GTP/GDP-binding proteins. Lanes incubated with radioactive ATP did not produce an autoradiographic signal. Although the peroxisomal membrane contains a 70 kDa member of the P-glycoprotein-related ATP-binding protein superfamily [32], it is known that these proteins do not retain their ATP-binding capacity after denaturation. For the sake of comparison, Fig. 2 also shows GTP-binding to membrane proteins obtained from pyrophosphate-treated microsomes (endoplasmic reticulum fragments). Endoplasmic reticulum contains small GTP-binding proteins [6]. GTP-binding was observed in the same molecular mass region as with peroxisomal membranes. Although the same amount of membrane proteins was used for peroxisomes and microsomes, the peroxisomal signal was the stronger one, making it unlikely that GTP-binding to the peroxisomal membrane proteins was the result of contamination with microsomal membranes.

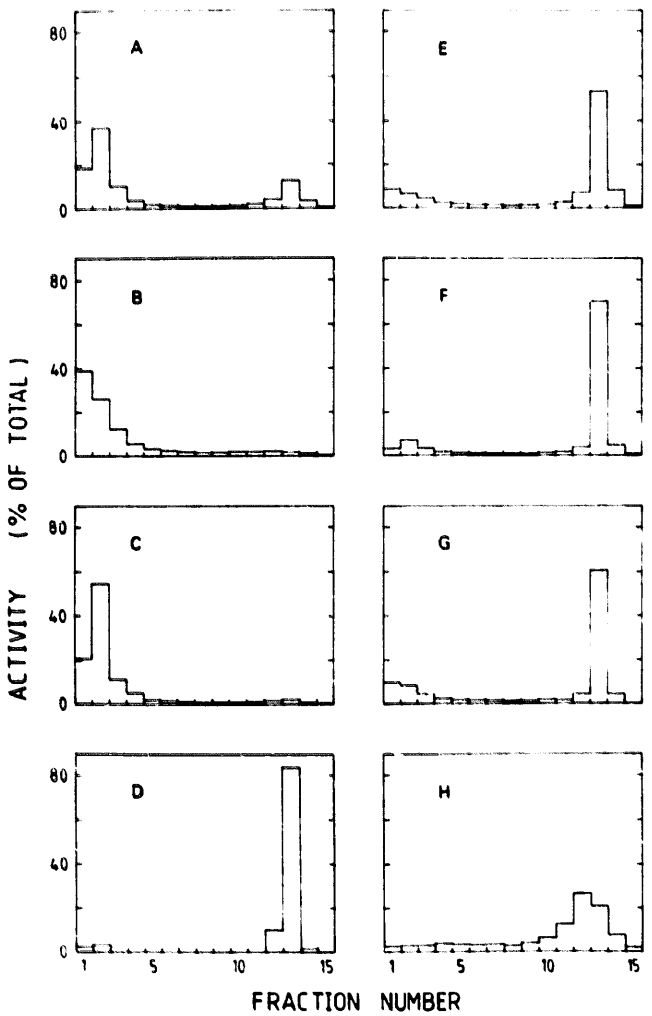

Fig. 1. Subfractionation of a λ-fraction on a self-generating Percoll gradient. A λ-fraction, prepared by differential centrifugation and enriched in peroxisomes, was subfractionated by isopycnic centrifugation in an isoosmotic self-generating Percoll gradient. The gradient fractions were analyzed for protein (A), acid phosphatase (lysosomes; B), glutamate dehydrogenase (mitochondria; C), galactosyltransferase (Golgi, D), 5'-nucleotidase (plasma membrane, E), glucose-6-phosphatase (endoplasmic reticulum; F), esterase (endoplasmic reticulum; G) and catalase (peroxisomes; H). Results are expressed as percentage of total gradient activity or content present in each fraction numbers on the abscissa. Fractions 1 and 15 represent the fractions of highest and lowest density, respectively. Recoveries were between 70 and 101%.

In order to further exclude that GTP-binding to peroxisomal membranes was the result of contaminating organelles, Percoll-purified peroxisomes were subfractionated on a discontinuous Nycodenz gradient. Fig. 3 shows the gradient distribution of marker enzymes and of GTP-binding quantified by densitometry. Fig. 3 shows that GTP-binding closely followed the distribution of the peroxisomal marker catalase and not those of markers for other cell compartments known to contain small GTP-binding proteins (glucose-6-phosphatase and esterase: endoplasmic reticulum; galactosyltransferase: Golgi; 5'-nucleotidase: plasma membrane). These data indicate that at least the major portion of GTP-binding to Percoll-purified peroxisomal fractions is due to binding to the peroxisomal membrane itself and not to binding to contaminating organelles.

Percoll-purified peroxisomes and microsomes prepared by differential centrifugation were then subjected to carbonate treatment, which releases not only the matrix (luminal) proteins, but also the peripheral membrane proteins. GTP-binding was studied in the carbonate-treated membranes and compared with that in pyrophosphate-treated membranes. GTP-binding was equally strong in carbonate- and pyrophosphate-treated membranes both for peroxisomes and microsomes and no GTP-binding could be detected in the protein fractions that were released by either treatment (data not shown). This indicates that the GTP-binding proteins are firmly anchored in the peroxisomal membrane, possibly by an isoprenyl moiety, as has

TABLE I

Composition of the λ-fraction and the Percoll-purified peroxisomal fraction

Results are expressed as percentages of total homogenate activity and relative specific activities. Relative specific activity is defined as the percentage of total homogenate activity present in a particular fraction divided by the corresponding percentage of total homogenate protein. It indicates the purification factor calculated on a protein basis. The results of a representative experiment are shown.

Enzyme	% of total homogenate activity		Relative specific activity	
	λ-fraction	Percoll-purified fraction	λ-fraction	Percoll-purified fraction
Catalase	21.8	4.9	2.8	16.2
Glucose-6-phosphatase	4.9	0.09	0.6	0.3
Esterase	11.0	0.16	1.4	0.5
Galactosyltransferase	5.7	0.13	0.7	0.4
5'-Nucleotidase	1.5	0.08	0.2	0.2
Acid phosphatase	42.0	1.1	5.3	3.8
Glutamate dehydrogenase	16.9	0.24	2.1	0.8
Protein	7.9	0.3	-	-

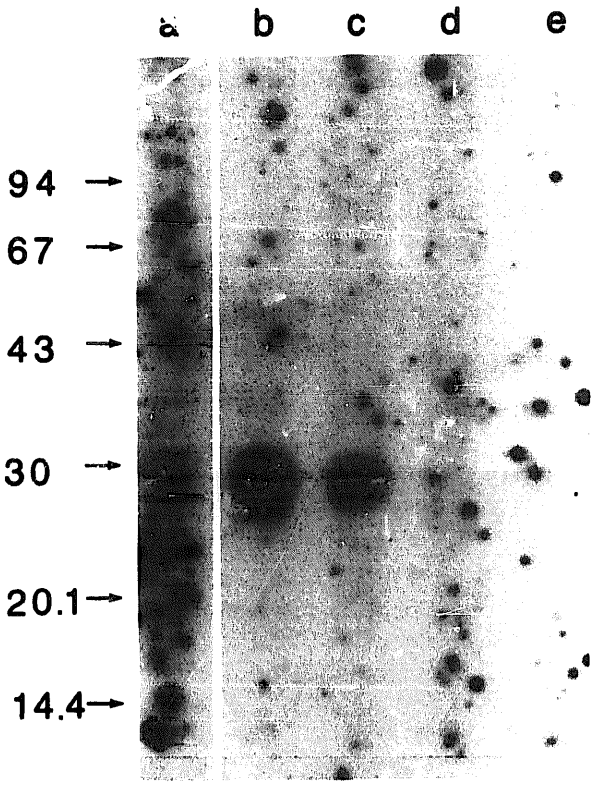

Fig. 2. GTP-binding proteins in the peroxisomal membrane. Membranes were obtained by pyrophosphate treatment of microsomes prepared by differential centrifugation and of Percoll-purified peroxisomes. The membrane proteins were solubilized, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (200 μg of membrane protein in each lane) and blotted onto nitrocellulose sheets which were incubated with 1 μCi/ml [α-^{32}P]GTP or [α-^{32}P]ATP (concn.: 0.6 nM). The blots were autoradiographed for 120 h. Lane a: microsomal proteins incubated with radioactive GTP; lanes b–d: peroxisomal proteins incubated with radioactive GTP in the absence (b) or presence of 10 μM unlabelled GMP (c) and 10 μM unlabelled GDP (d); lane e: peroxisomal proteins incubated in the presence of radioactive ATP. Molecular masses expressed in kDa are indicated on the left side.

been described for a number of these proteins [33–35].

Next, the specificity for GTP-binding was studied in carbonate-treated membranes from Percoll-purified peroxisomes and from microsomes. As evident from Fig. 4, radioactive GTP-binding was abolished by the same concentration range of unlabelled GTP in peroxisomal and microsomal membrane-protein fractions. ATP used at similar concentrations did not affect GTP-binding. With peroxisomes, three distinct bands with molecular masses of 29, 27 and 25 kDa, respectively, were discerned. The 29 kDa band was most abundant. Microsomes revealed two clear bands of 30 and 26 kDa, respectively. Here, the 26 kDa band was most abundant. (It should be noted that the molecular mass estimations – see legend to Fig. 4 – do not necessarily indicate that the molecular masses of the microsomal GTP-binding proteins are different from those of their peroxisomal counterparts). Labelled GTP-binding to peroxisomal membrane proteins was also abolished by the GTP-analogs GTPγS and p[NH]ppG. CTP and UTP, like ATP, did not diminish GTP-binding (Fig. 5). Identical results were obtained with microsomes (data not shown).

Since small GTP-binding proteins are generally located on the cytoplasmic side of the organelle membrane [6,8,11], we incubated intact peroxisomes with trypsin concentrations that did not affect the latency of catalase (data not shown) and, therefore, the permeability of the peroxisomal membrane. Trypsin treatment markedly decreased GTP-binding to the 29, 27 and 25 kDa polypeptides but led to the appearance of GTP binding to a 23 kDa polypeptide, possibly a proteolytic degradation product of one of the GTP-binding proteins (Fig. 6). The decrease in GTP-binding after trypsin treatment indicates that the peroxisomal GTP-binding proteins are exposed to the cytosol as is the case in other cell organelles.

Finally, we investigated whether pretreatment of rats with a peroxisome proliferator would increase

GTP binding to the peroxisomal membrane. Peroxisome proliferators induce a number of peroxisomal enzymes (e.g., the β-oxidation enzymes) 10- to 20-fold. Other enzymes are only slightly (e.g., catalase; 1.5-fold) or not (e.g. urate oxidase) increased (see Ref. 36). GTP-binding was studied in Nycodenz fractions (Fig. 3) containing the highest catalase activity, from control and clofibrate-treated rats. Quantification of GTP-binding by densitometry revealed that, expressed per unit of catalase, peroxisomal fractions from clofibrate-treated rats bound 29 ± 8% (mean ± S.E.; $n = 4$) more GTP than fractions derived from control rats. Since catalase, expressed per g of liver, is increased by approx. 50% after clofibrate treatment, these data indicate that the peroxisomal GTP-binding proteins are 2-fold increased, when expressed per g of tissue, after treatment with the peroxisome proliferator. Since clofibrate treatment causes a 40% liver enlargement, the increase in peroxisomal GTP-binding per whole liver would be 2.7-fold.

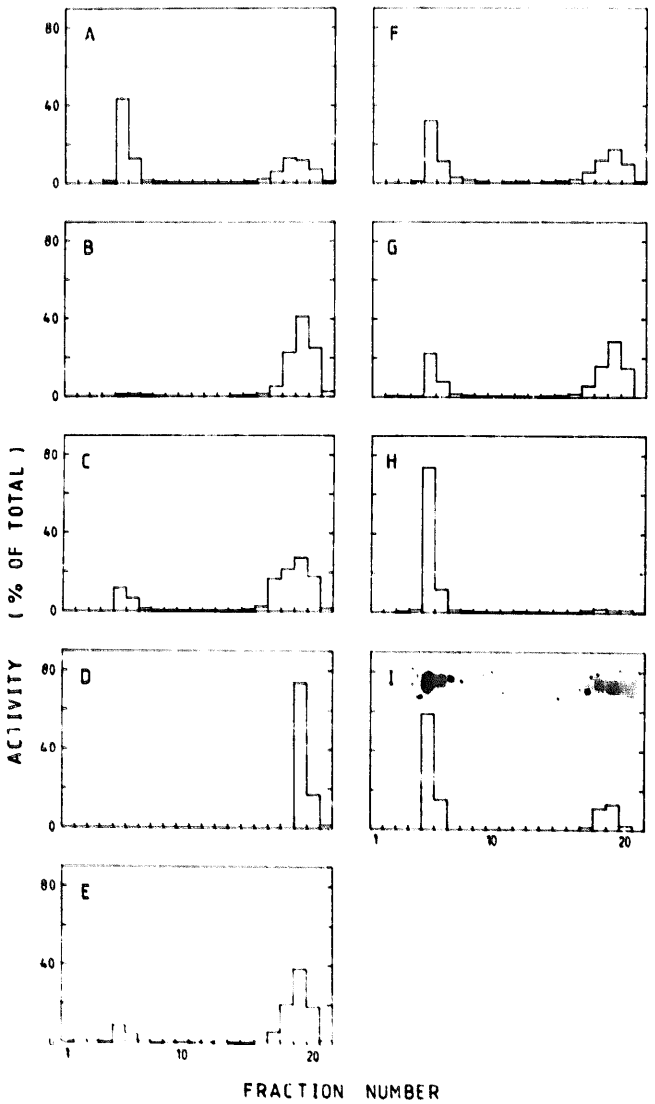

Fig. 3. Subfractionation of a Percoll-purified peroxisomal fraction on a Nycodenz gradient. A Percoll-purified peroxisomal fraction was subfractionated by centrifugation through a discontinuous Nycodenz gradient. The gradient fractions were analyzed for protein (A), acid phosphatase (B), glutamate dehydrogenase (C), galactosyltransferase (D), 5'-nucleotidase (E), glucose-6-phosphatase (F), esterase (G), catalase (H) and GTP-binding (I), quantified by densitometric scanning of the autoradiograph (I, inset). Results are expressed as percentage of total gradient activity or content present in each fraction, numbered on the abscissa. Fractions 1 and 21 represent the bottom and top fractions, respectively. Recoveries for protein and marker enzymes were between 77 and 99%.

Gel electrophoresis of proteins from carbonate-treated peroxisomal membranes (Nycodenz fraction 5, see Fig. 3) revealed the major peroxisomal integral membrane proteins (70, 49, 36, 22 and 15 kDa) [37,38], but only two faint bands could be observed in the GTP-binding molecular mass region (Fig. 7), demonstrating that the GTP-binding proteins are only minor components of the peroxisomal membrane.

Discussion

The present work shows that the peroxisomal membrane contains three small GTP-binding proteins. They are firmly anchored in the membrane, possibly by an isoprenyl moiety [33–35], and they are exposed to the cytosol like the small GTP-binding proteins in other cell organelles [6,8,11].

Peroxisomal GTP-binding was only slightly increased after treatment of the animals with a peroxisome proliferator. It is an open question whether this increase is the result of an induction of the peroxisomal GTP-binding proteins, as is the case for a number of other peroxisomal proteins (see Ref. 36), or of an increase in membrane association of the proteins, since GTP-binding proteins can exist in soluble and membrane-bound forms [12,16].

Evidently, subcellular fractionation cannot exclude with absolute certainty that a particular enzyme or protein is located in a contaminating organelle that co-purifies with the organelle under study. We consider this possibility unlikely. First, marker enzyme analysis ruled out that GTP-binding was due to contaminating endoplasmic reticulum, plasma membranes or Golgi apparatus, the major cellular membrane systems known to contain small GTP binding proteins [6–8]. Second, significant contamination of our peroxisomal fractions with vesicles of the exocytotic/endocytotic pathways is also unlikely, since these vesicles are not abundant in the cell and since these vesicles (or other unsuspected cell organelles) most probably do not have the same mass, density and permeability characteristics as peroxisomes. Our purification procedure was based on a combination of differential centrifugation (mass) and density centrifugation. In addition, the density of peroxisomes in different media is co-determined by the permeability of the

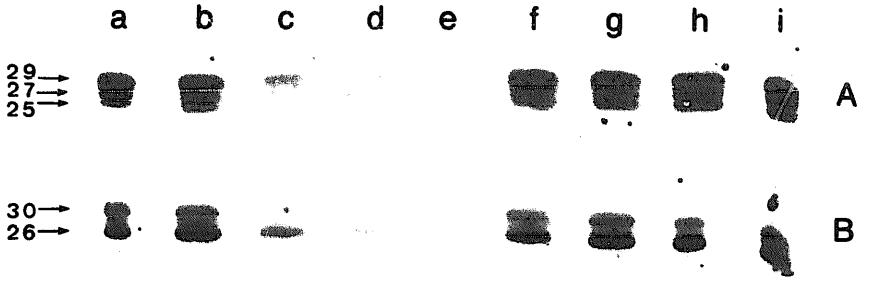

Fig. 4. Displacement of bound radioactive GTP by unlabelled GTP and ATP. Membranes were obtained by carbonate treatment of Percoll-purified peroxisomes and microsomes. The membrane proteins were separated by electrophoresis (200 μg of protein in each lane) and blotted onto nitrocellulose sheets, which were incubated with 1 μCi/ml [α-^{32}P]GTP (concn.: 0.3 nM) and then autoradiographed for 20 h. A: Peroxisomal proteins incubated in the absence (a) or presence of 1 nM (b), 5 nM (c), 15 nM (d) and 50 nM (e) unlabelled GTP or 1 nM (f), 5 nM (g), 15 nM (h) and 50 nM (i) unlabelled ATP. B: Microsomal proteins incubated under the same conditions as described for A. Molecular masses, expressed in kDa are shown on the left side. Molecular masses for the peroxisomal GTP-binding proteins were 29.5 ± 0.7 kDa, 27.6 ± 0.8 kDa and 25.4 ± 0.7 kDa (means ± S.E.; n = 7); those for the microsomal proteins were 30.1 ± 0.4 kDa and 26.6 ± 0.5 kDa (means ± S.E.; n = 4).

Fig. 5. Displacement of bound radioactive GTP by unlabelled nucleotides and analogs. Nitrocellulose blots of peroxisomal membrane proteins obtained as described in the legend to Fig. 4 were incubated in the presence of 1 μCi/ml [α-^{32}P]GTP (concn.: 0.4 nM) and in the absence (a) or presence of 50 nM unlabelled GTP (b), GMP (c), GDP (d), GTPγS (e), p[NH]ppG (f), ATP (g), CTP (h) and UTP (i) and then autoradiographed for 20 h. Molecular masses expressed in kDa are shown on the left side.

peroxisomal membrane. The peroxisomal membrane is permeable to small water-soluble molecules, because of the presence of a proteinaceous large conductance-channel [21,39]. This non-selective permeability is an unusual property for a biological membrane that is shared only by the mitochondrial outer membrane in animal cells. Percoll particles are too large to enter peroxisomes but Nycodenz does, so that peroxisomes acquire a higher density in Nycodenz than in Percoll media. This difference in density is not observed with impermeable organelles, so that the chance of copurifi-

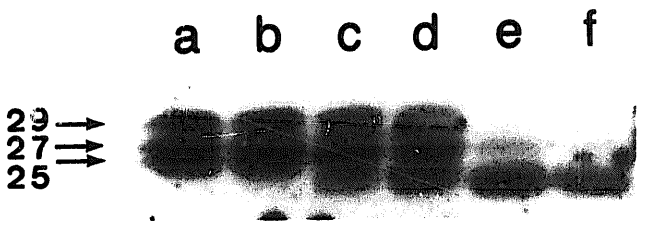

Fig. 6. Effect of trypsin treatment of intact peroxisomes on radioactive GTP-binding. Peroxisomes from a Nycodenz gradient (fraction 5 in Fig. 3) were incubated for 10 min at 30°C in the absence (a) or presence of 0.1 μg/ml (b), 0.5 μg/ml (c), 1 μg/ml (d), 10 μg/ml (e) and 100 μg/ml (f) trypsin. Peroxisomal protein concentration was 2.2 mg/ml. Incubations were terminated by boiling in electrophoresis sample buffer and the denatured proteins were separated by electrophoresis and blotted onto nitrocellulose sheets, which were incubated with 1 μCi/ml [α-^{32}P]GTP (concn.: 0.6 nM) and autoradiographed for 72 h. Molecular masses expressed in kDa are shown on the left side.

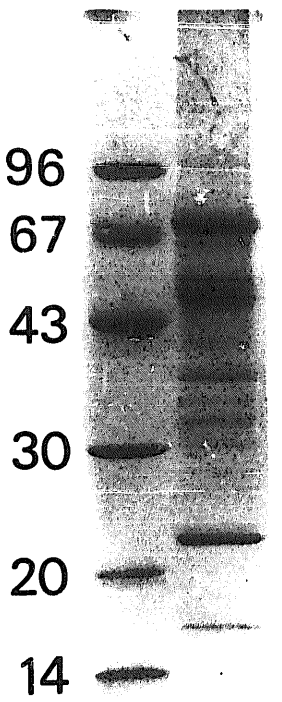

Fig. 7. Peroxisomal integral membrane proteins. Membranes were prepared by carbonate treatment of peroxisomes from a Nycodenz gradient (fraction 5 in Fig. 3) and the membrane proteins (50 μg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and stained with Coomassie blue (right lane). The left lane shows the position of the marker proteins. Their molecular mass is expressed in kDa.

cation of peroxisomes and another organelle in combined Percoll and Nycodenz media becomes extremely small.

The function of the peroxisomal GTP-binding proteins remains unknown. It is generally believed that peroxisomes multiply by budding or fission [17]. Recently, peroxisome-forming sheets were described in mouse hepatocytes cultured in the presence of a peroxisome proliferator [19]. The peroxisome-forming sheets are membranous structures from which peroxisomes are budding. Since small GTP-binding proteins have been implicated in fusion/fission processes in the exocytotic/endocytotic pathways, we speculate that the peroxisomal GTP-binding proteins are involved in the control of peroxisome multiplication and, as a consequence, in peroxisome biogenesis and proliferation.

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